Probiotics and Antimicrobial Proteins (2020) 12:1370–1384
https://doi.org/10.1007/s12602-020-09645-8

Effects of *Lactobacillus rhamnosus* ATCC 7469 on Different Parameters Related to Health Status of Rainbow Trout (*Oncorhynchus mykiss*) and the Protection Against *Yersinia ruckeri*

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Published online: 3 April 2020

Abstract

In the current study, we investigated the effect of a probiotic bacterium (*Lactobacillus rhamnosus* ATCC 7469) microencapsulated with alginate and hi-maize starch and coated with chitosan on improving growth factors, body composition, blood chemistry, and the immune response of rainbow trout (initial weight: 18.41 ± 0.32 g). Four experimental diets were formulated to feed fish for 60 days. They were control diet without any additive (C), diet added with beads without probiotic (E), a probiotic sprayed to the diet (L.r), and encapsulated probiotic supplemented diet (E-L.r). The results indicated that feeding with E-Lr significantly improved weight gain (84.98 g) and feed conversion ratio (0.95) compared to the other groups ($P < 0.05$). Also, fish fed E-Lr diet had a significantly higher value of whole-body protein (17.51%), total protein in the blood (4.98 g/dL), lysozyme (30.66 U/mL), alternative complement pathway hemolytic activity (134 U/mL), superoxide dismutase (203 U/mg protein), and catalase (528.33 U/mg protein) ($P < 0.05$) as compared to those fed the control diet. Similarly, a higher relative expression of immune-related genes such as interleukin-1 (Il-1) and tumor necrosis factor-alpha (TNF-1α) were reported in those fed E-L.r and L.r diets respectively. Interestingly, the fish fed dietary E-L.r had a significantly lower value of lipid in the whole body (4.82%) and cholesterol in the blood (160.67%) in comparison with those fed the control diet ($P < 0.05$). At the end of the experiment, all groups were challenged by *Yersinia ruckeri* where the survival rate of rainbow trout fed dietary E-L.r (70.36%) was statistically higher than that of the others ($P < 0.05$). Overall, the results suggested that encapsulated probiotic *Lact. rhamnosus* ATCC 7469 acted better than unencapsulated probiotic and has a potential to improve growth performance, flesh quality, and the immune response of rainbow trout.

Keywords Immunostimulant · *Lactobacillus rhamnosus* · ATCC 7469 · Probiotic · Immunity · Hypocholesterolemic effect · *Yersinia ruckeri*

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Introduction

Increasing population and the need to provide food for them has attracted the attention of the different countries to increase the number of aquaculture products in their food basket. Furthermore, the high quality of aquaculture products for human nutrition has made the aquaculture industry the fast growing sector of food industry with 8% growing each year [1]. Farmers tend to increase the stocking density in some species such as rainbow trout (*Oncorhynchus mykiss*) in order to boost the fish production. However, high density adversely affects health, survival ratio, growth performance, and product quality of rainbow trout [2, 3]. One of the worst consequences of high density has been an increase in the population of pathogenic microorganisms. In the last decades, irresponsible use of antibiotics for killing these microorganisms has brought the emergence of numerous resistant bacteria [4, 5]. In this sense,

supplementing fish diets with some alternative immunostimulants, such as probiotic for boosting the immune system, are an appropriate substitution. Probiotics can help to replace or minimize the application of antibiotics and chemotherapeutics in fish farming [6–8]. The inclusion of probiotics in the diets is a key step to protect farmed fish against stress or infection under farming conditions [9]. Probiotics benefit the host by improving disease resistance, health status, growth performance, feed utilization, and stress response when administered in the feed or in the rearing water [10]. Among probiotics, different strains of *Lactobacillus* spp. have been added to farm animals' diet for a long time and still is one of the most common probiotics in animal farming [11]. Different strains of *Lactobacillus rhamnosus* had various health effects such as the prevention of acute diarrhea, allergies, and lowering cholesterol levels and immune stimulation in humans [12]. In aquaculture studies, some investigators reported the potential of different *Lact. rhamnosus* strains administration against *Aeromonas salmonicida*, *Vibrio anguillarum*, and *Flavobacterium psychrophilum* in rainbow trout [13]; and *Edwardsiella tarda* [14] and *Streptococcus agalactiae* [15] in tilapia (*Oreochromis niloticus*). Many studies also indicated the role of different strains of *Lact. rhamnosus* in improving the growth performance and the immune response in different fish species [16–22]. However, there is no study investigating the effect of *Lact. rhamnosus* ATCC 7469 on aquaculture species.

Although probiotics such as *Lact. rhamnosus* ATCC 7469 provide a healthy condition for the host, it is important to supplement in appropriate ratios [23, 24]. It was recommended that minimum level of probiotic in food product to be10^6 colony-forming unit gram^{-1} (CFU/g) [25], or 10^7 CFU/g at the point of delivery or to be eaten in appropriate quantities (daily intake of 10^8–10^9 cells) [26]. Most probiotics species such as *Lact. rhamnosus* ATCC 7469 can be destroyed by harsh processing and gastrointestinal conditions, such as low pH, salinity, temperature, or oxidative stress [27]. Protecting probiotic living cells against adverse environmental conditions using a physical barrier is an approach currently receiving great considerations in order to maintain the viability of probiotics during the preparation, storage, and consumption stages [28, 29]. The encapsulation technique is a simple, safe, and reliable method, which increases the survival of bacteria. This method also results in the controlled release of probiotics at the targeted region (posterior intestinal tract, as they are supplied orally) [30, 31].

Previous investigators found that coating alginate microcapsules with chitosan improves the stability of alginate beads and thus the viability of encapsulated probiotic organisms up to 80–95% [32, 33]. Regardless of probiotic compounds, available materials in capsules such as starch and oligosaccharides promote the survival of probiotic bacteria as well [33, 34].

Rainbow trout with 814 thousand tonnes annual production has been considered one of the most important inland aquaculture species worldwide, especially in Chile, Turkey, and Iran [1]. According to Iran Fishery Organization statistic in 2016, rainbow trout production was 166 thousand tones, being the highest consumed species nationally and it is predicted to reach 200 thousand tones soon.

Although probiotics are widely used in aquaculture, a few pieces of research have been performed on encapsulating probiotics for aquaculture application [15, 29, 35–39]. According to our knowledge, there is no study about the effect of *Lactobacillus* species encapsulation on growth performance, body composition, immunity, and resistance against pathogens in aquaculture species. Also, there is no study investigating the effect of supplementing *Lact. rhamnosus* ATCC 7469 on aquaculture research. To get more insight into our project about the effect of probiotic on finfishes [Montazeri et al. 2019 (unpublished), 40], we designed this research. Hence, the purpose of this work is to assess the effect of probiotic *Lact. rhamnosus* ATCC 7469 administered in two different ways on growth performance, body composition, immune response, blood constituents, and disease resistance against *Yersinia ruckeri* in rainbow trout.

Materials and Methods

We declare that all steps of this experiment were performed according to the Tarbiat Modares University protocols (The guidelines, adopted from the Declaration of Helsinki (1975) and The Society for Neuroscience Animal Care and Use guidelines (1998)) for supporting animal ethics. This guideline was approved for implementation by the Medical Ethics Committee, School of Medical Sciences of the Tarbiat Modares University on 18 April, 1385/17th April, 2006. This protocol was mentioned in our previous works [41–44].

Probiotic Preparation and Encapsulation

Pure freeze-dried probiotic culture of *Lact. rhamnosus* (ATCC 7469) was purchased from Persian Type Culture Collection and was cultured in the MRS-broth (de Man-Rogosa-Sharpe) at 37 °C for 48 h. The probiotic biomass was collected by centrifugation at 4000 g for 10 min at 4 °C. Then, it was washed twice with sterile saline 0.9% and collected with the same centrifugation conditions. The cell count was determined by decimal series dilution and plating on MRS agar. Culture cells were preserved with 30% glycerol at − 20 °C until the usage. All glassware and solutions used in the protocols were sterilized at 121 °C for 15 min. The microcapsule was produced by meaning the previously described emulsification method with some modifications [45, 46]. A 2%

sodium alginate (medium viscosity, Sigma–Aldrich, St. Louis, USA) and 1% resistant corn starch (Sisco Research Laboratories Pvt. Ltd., India) were mixed in distilled water and then 1% of probiotic (10^8 CFU/mL) was added to the mixture. Next, the produced mixture was dispersed in 150 mL canola oil containing 1.5% TWEEN 80 at 400 rpm for 5 min using a magnetic stirrer (BOECO, Germany). It was followed by adding 150 mL of 0.5 M calcium chloride solution. Then, precipitation of calcium-alginate beads at the bottom of the beaker at the calcium chloride layer (water phase) was done by keeping the mixture stable for 25–30 min. They were harvested by centrifugation (750 g, 5 min) and further washed with 0.9% saline containing 5% glycerol and stored at 4 °C. Alginate beads were coated with chitosan according to previous methods [47]. In brief, 0.4 g chitosan (448869, low molecular weight, Sigma-Aldrich, St. Louis, USA) was dissolved in 90 mL distilled water acidified with glacial acetic acid to achieve a final chitosan concentration of 0.4% (*w*/*v*). The chitosan solution was autoclaved at 121 °C for 15 min. Then, the pH was adjusted to 5.7 by adding 1 M NaOH. The solution was filtered (Whatman no. 4). Alginate beads were washed with distilled water, immersed in 100 mL of chitosan solution, and gently stirred at 100 rpm for 40 min in an orbital shaker. Finally, the chitosan-coated beads were washed with distilled water and used on the same day.

Fish and Fish Rearing

The Rainbow trout ($n = 180$, with an average weight 18.41 ± 0.32 g and average length 12.37 ± 0.22 cm) were purchased from a private farm (Babol, Mazandaran, Iran) and transferred to the Nutrition Laboratory in Faculty of Natural Resources and Marine Sciences at Tarbiat Modares University. Before starting the experiment, acclimatization of rainbow trout was done for 15 days after which they randomly were distributed into a semi-recirculation system with 12 fiberglass circular tanks (300-L, 15 fish per tank). The fish were fed twice daily with experimental diets at satiation levels. Daily, 30–40% of the tank water was exchanged in order to keep all tanks clean. We adjusted a 12 h:12 h light:dark photoperiod for 60 days. Water temperature, dissolved oxygen, and pH were 16 ± 1.5 °C, 7.0 ± 1.6 mg/L, and 7–8, respectively. Chemical factors in water were measured as follows: temperature by mercury thermometer (Zomorodazma Company, Iran), dissolved oxygen by Cyberscan Eutech instruments (DO 110, Singapore), and pH by Hanna instrument (8314, USA). The level of total ammonia-nitrogen was measured every 2 weeks which was lower than 0.05 mg/L [43].

Feed and Feeding

The basal diet was formulated with 50% fishmeal as the protein source (Table 1). The feeding trial was conducted in four experimental groups with three replicas each. The treatments included control (C), capsule without probiotic (E), sprayed probiotic to the diet (L.r), and encapsulated probiotic supplemented diet (E-L.r). Free probiotic with a concentration of 10^9 CFU/mL was sprayed with canola oil to the 100-g diet, mixed manually, and air dried on a clean bench for 24 h (to reach approximately 10^8 CFU/100 g). The encapsulated

Table 1 Composition of the different diets used in the experiment: control basal diet (C), diet with empty microcapsules added (E), diet sprayed with *Lactobacillus rhamnosus* ATCC 7469 (L.r.), and diet with *Lact. rhamnosus* encapsulated added (E-L. r.)

Ingredients	Control	E	L	EL
	g per 100 g diet			
Fish meal[a]	50.00	50.00	50.00	50.00
Soybean meal[a]	21.23	21.23	21.23	21.23
Wheat flour	10.00	10.00	10.00	10.00
Dextrin[b]	3.00	3.00	3.00	3.00
Fish oil[a]	4.50	4.50	4.50	4.50
Soybean oil	4.50	3.50	3.50	3.50
Soybean lecithin[c]	0.50	1.00	1.00	1.00
Vitamin premix[d]	2.00	2.00	2.00	2.00
Mineral premix[e]	2.50	2.50	2.50	2.50
Antifungus[a]	0.25	0.25	0.25	0.25
Dicalcium phosphate[a]	0.50	0.50	0.50	0.50
Antioxidant (BHT) [f]	0.02	0.02	0.02	0.02
Filler[b]	1.00	0.00	0.90	0.00
Beads of capsule	0.00	1.00	0.00	0.00
Free probiotic	0.00	0.00	0.10	0.00
Encapsulated probiotic	0.00	0.00	0.00	1.00
Total	100	100	100	100
Proximate composition (%)				
Crude protein	47.22	47.53	47.69	47.04
Crude lipid	16.44	16.53	16.79	16.46
Ash	11.22	11.00	11.43	11.02
Carbohydrate[g]	25.13	25.46	24.96	25.61
Gross energy (kJ/g diet) [h]	21.85	21.78	21.96	21.59

[a] Khorak-dam Abzian corporation, Sari, Iran

[b] Merck KGaA, Darmstadt, Germany

[c] Soybean lecithin with phosphatidylcholine (Behpak company, Iran)

[d] Science Laboratories Company (Qazvin, Iran). Each 1000 g of vitamin premix Contains: vit. A 1600000 IU, vit. D_3 400,000 IU, vit. E 30 g, thiamin 10 g, riboflavin 8 g, pyridoxine 40 g, vit. B_9 3 g, cyanocobalamin 0.01 g, vit. C100 g, vit. k_3 10 g, biotin 10 g

[e] Science Laboratories Company (Qazvin, Iran). Each 1000 g of mineral premix Contains: Fe 20 g, Zn 60 g, Se 400 mg, Co 200 mg, Cu 2 g, Mn 40 g, I 400 mg

[f] Antioxidant (Gluba Toxi, French)

[g] Carbohydrate calculated based on the formula: 100-(crude protein + crude lipid + ash)

[h] Estimated gross energy was calculated based on 1 g crude protein being 23.6 kj, 1 g crude fat being 39.5 kj, and 1 g carbohydrate being 17.2 kj [86]

probiotic was added to the basal diet in the same way as for the unencapsulated. To establish the same conditions, the control diet was sprayed with distilled water and canola oil. During the 60 days of experimental period, the diets including different forms of probiotics were prepared every 2 weeks. For protecting the diets from spoilage, all diets were stored at 4 °C. To assess the viability of the sprayed bacterium, 1-g L.r diet was dissolved, and its bacterial count was performed on MRS agar at different time intervals (0, 5, 10, and 15 days).

Growth Performance and Sampling

At the end of the experiment, the fish were left fasted for 24 h and then anesthetized with clove oil stock solution (50 ppm). The body weight and length of each fish were measured. Growth parameters—weight gain (WG), specific growth rate (SGR), and feed conversion ratio (FCR)—were calculated using the common equations as follows:

$$\text{Wet weight gain (WG)} : (\text{g}) = (\text{final mean wet weight (g)} - \text{initial mean wet weight (g)}).$$

$$\text{Specific growth rate (SGR)} : \%/\text{day} = 100 * \left((\ln \text{final wet weight} - \ln \text{initial wet weight}) / \text{duration (day)}.\right.$$

$$\text{Feed conversion ratio (FCR)} = \text{consumed feed by fish during the study period (g)} / (\text{final body weight (g)} - \text{initial body weight (g)}).$$

Biochemical Analysis

Crude protein, crude lipid, moisture, and ash contents in the diets and whole body of rainbow trout were determined according to the standard methods of AOAC (2005) [48]. For more details about the standard methods and instruments utilized, please check our previous papers [41–43]. Liver superoxide dismutase (SOD) and catalase (CAT) activity were assayed using commercial kits (ZEIIBio, GmbH, Germany), according to the mentioned methods in kits.

Immunological Analysis

Collection of Blood and Blood Biochemistry

Heparinized syringes were used to collect blood from the caudal vein. The plasma was obtained through centrifugation at 1500 g for 5 min at 4 °C and kept in a freezer at − 80 °C until subsequent analysis. Plasma samples were thawed and the biochemical indices including total proteins (g/dL), albumin (g/dL), cholesterol (mg/dL), triglycerides (mg/dL), and glucose (mg/dL) were measured using commercial spectrophotometric kits (Pars Azmun Co. Ltd., Iran) by a chemistry analyzer (model AE 600, ERMA, Tokyo, Japan). Three fish from each tank were randomly taken for analyzing blood biochemistry and immune parameters.

Alternative Complement Pathway Hemolytic Activity (ACH50)

The ACH50 was assayed by hemolysis of rabbit red cells (RaRBC) [49]. Briefly, a series of volumes of diluted plasma ranging from 0.1 to 0.25 mL was dispensed to test tubes where the total volume was brought up to 0.25 mL with a barbitone buffer in the presence of ethylene glycol-bis (2-aminoethoxy)-tetraacetic acid (EGTA), and Mg^{+2}. Then, 0.1 mL of RaRBC was added to each tube. After incubation for 2 h at 22 °C, 3.15 mL of 0.9% NaCl was added. In the next step, the sample was centrifuged at 836 g for 5 min at 4 °C to eliminate unlysed RaRBC. The absorbance of the supernatant was measured at 414 nm. Finally, the volume yielding 50% hemolysis was used for determining the complement activity of the sample as follows:

$$\text{ACH50 value (U/mL)} = 1/(K \times (\text{reciprocal of the serum dilution}) \times 0.5)$$

In the above relation, K represents the volume of serum in mL, which causes 50% hemolysis, 0.5 is constant, and finally the dilution factor in this test is 0.01.

Lysozyme Activity Assay

Lysozyme activity was determined in plasma through measuring (530 nm after 1 and 5 min at 22 °C) the reduction of the turbidity of a *Micrococcus lysodeikticus* suspension, as previously described [50]. Lysozyme activity was determined using as standard lyophilized hen egg-white lysozyme (Sigma-Aldrich, St. Louis, USA) serially diluted in a sodium phosphate buffer (0.05 M; pH 6.2). A unit of enzyme activity was defined as a reduction in absorbance by 0.001/min.

Plasma Immunoglobulin M Assay

Total immunoglobulin (IgM) levels were measured by using an enzyme-linked immunosorbent assay (ELISA) mouse kit purchased from Eastbiopharm Company (Hangzhou Eastbiopharm Co., Ltd. China). Briefly, wells of flat-bottomed 96-well plates were coated with trout plasma samples and washed with wash buffer (20× (PBS with 1% Tween™ 20)) to eliminate the excess of Ag. Then, a biotinylated detection antibody specific for mouse Ig M and avidin-Horseradish Peroxidase (HRP) conjugate were successively added to each well and incubated at 37 °C for 1 h. Free components were washed away. The HRP substrate

(tetramethylbenzidine) solution was added to each well and the plate was incubated at 37 °C for 1 h. The oxidation of tetramethylbenzidine by peroxidase develops a blue color in wells. The enzyme-substrate reaction was terminated by the addition of stop solution and the color turned yellow. The optical density (OD) was measured spectrophotometrically at a wavelength of 450 nm in a plate reader (model Epoch 2, Biotek, USA). Blank wells were chromogen solutions and stop solution. The mean absorbance of negative controls for each plate was subtracted from the optical density (OD) at 450 nm. The OD value is proportional to the concentration of mouse Ig M and we calculated the concentration of Ig M in the samples by comparing the OD of the samples to the standard curve.

Real-Time RT-PCR

At the end of the experimental period, 50 to 100 mg of kidney tissue was collected and immediately stored in liquid nitrogen. Then, theses samples were transferred to − 80 °C until RNA extraction. Total RNA extraction was performed according to Biozol Reagent company protocol (Bio flux; China). The quality and quantity of total RNA were measured by 1% agarose gel electrophoresis and NanoDrop spectrophotometer at 280 and 260 nm (Thermo Fisher Scientific, USA). The first-strand cDNA was synthesized using SuPrime Script RT Premix (2×) cDNA Synthesis kit (GeNet BIO Inc.; Daejeon, South Korea) by following the manufacturer's protocol. Primers for target genes (TNF-1α and IL-1) and reference gene (β-Actin) in rainbow trout had already been designed (Table 2). The expression of target genes was examined using SYBR Green Kit (Bio Pars Cyber, Iran) on iQ5 System (Bio-Rad, USA). The complete method was described by Miandare et al. (2016) [51].

Bacterial Challenge by *Y. ruckeri*

At the end of the experimental trial, *Y. ruckeri* bacterial challenge was done. For this purpose, bacteria were grown in tryptic soy broth (TSB; QUELAB) for 24 h at 22 °C, centrifuged at 6000 g for 10 min, and suspended in sterile PBS. In this way, 18 fish per treatment (6 fish per tank) were intraperitoneally (IP) challenged with 0.1 mL per fish of bacterial suspension (1×10^7 CFU/mL). The cumulative mortality (%) was recorded for 14 days once the challenge and any moribund fish were removed and bacteriologically examined to confirm the presence of the pathogen in their skin, liver, and kidney.

Statistical Analysis

The results are presented as the mean ± standard deviation (S.D). Statistical analysis was performed using SPSS (version 19). Normality of the data was assessed using the Kolmogorov-Smirnov test and the significant differences were determined using one-way analysis of variance (ANOVA). Duncan's test was applied for multiple comparisons**.** Differences were considered statistically significance when $P < 0.05$.

Result

Growth Performance

The growth performance of fish fed the experimental diets, supplemented or not with *Lact. rhamnosus* ATCC 7469, is presented in Table 3. According to these results, fish fed dietary E-L.r had a significantly higher value of WG (84.98 g) and SGR (2.78) compared to the other groups. On the other hand, rainbow trout fed E-L.r diet had a significantly lower value of FCR (0.95) as compared with those fed the control diet (1.34) ($P < 0.05$).

Body Composition

Table 4 reports the results of whole-body composition in rainbow trout fed diets supplemented or not with *Lact. rhamnosus*

Table 2 The sequences of primers used in the experiment

Target gene	Size of amplicon (bp)	GenBank ID	Primer sequence	Primer efficiency (%)
β-Actin	260	AF157514.1	F: ATGGAAGGTGAAATCGCC	99
			R: TGCCAGATCTTCTCCATG	
IL-1β1	323	NM_001124347	F: GATTCACAAGAACT AAGGAC	95
			R: ACTGTGATGTACTG CTGAAC	
TNF-α1	181	AJ277604.2	F: CAAGAGTTTGAACC TTGTTCAA	99
			R: GCTGCTGCCGCACA TAGAC	

Table 3 Growth performance parameters of rainbow trout fed different diets. Control basal diet (C), diet with empty microcapsules added (E), diet sprayed with *Lactobacillus rhamnosus* ATCC 7469 (L.r.), and diet with Lact. rhamnosus encapsulated added(E-L. r.)

	Control	E	L.r	E-L.r
Initial weight (g)	18.86 ± 0.92	19.16 ± 0.39	19.66 ± 0.55	19.72 ± 0.85
Final weight (g)	70.93 ± 5.80 c	74.83 ± 4.60 c	86.28 ± 6.91 b	104.70 ± 5.33 a
Weight gain (g)	52.07 ± 3.50 c	55.67 ± 4.21 c	66.62 ± 3.89 b	84.98 ± 4.40 a
SGR (% day^{-1})	2.19 ± 0.11 c	2.27 ± 0.23 bc	2.46 ± 0.41 b	2.78 ± 0.35 a
FCR	1.34 ± 0.66 b	1.19 ± 0.50 ab	1.08 ± 0.41 ab	0.95 ± 0.63 a
Survival rate (%)	100	100	100	100

Wet weight gain (WG): (g) = (final mean wet weight (g)–initial mean wet weight (g)

Specific growth rate (SGR): % day^{-1} = 100 (ln (final wet weight)–ln (initial wet weight))/duration (day)

Feed conversion ratio (FCR) = the weight of food consumed by fish during the study period (g) / (final body weight (g) − initial body weight (g))

Values are the means ± standard deviation of the all samples. Means without letter are not significantly different. Letters a, b, and c indicate significant differences in treatments, according to Duncan's multiple range tests ($P < 0.05$)

ATCC 7469. As observed, the fish fed dietary E-L.r had a significantly higher (17.51%) content of protein and a lower (4.82%) lipid content compared to the rainbow trout fed the control diet (15.75% and 6.75%) ($P < 0.05$). Also, there was no significant difference in ash and moisture contents in whole body of those fed the experimental diets.

Biochemical Blood Parameters

The biochemical parameters of blood in the fish fed experimental diets are presented in Table 5. According to the results, the fish fed E-L.r diet had a higher content of total proteins (4.98 g/dL) compared with those fed the control diet. Albumin, glucose, and triglyceride levels showed no significant difference between the treatments. Total cholesterol levels in the fish fed E-L.r (160.67 mg/dL) and L.r (156.5 mg/dL) diets were significantly lower than in those fed the control (185.00 mg/dL) and E (185.50 mg/dL) diets ($P < 0.05$).

Immune System Parameters and Gene Expression

Figure 1 demonstrates the results of immune system factors such as lysozyme, ACH50, and IgM of the fish fed experimental diets. Accordingly, diet supplementation with probiotic, in any way used, significantly improved the lysozyme and ACH50 activities ($P < 0.05$). Precisely, lysosome contents in the rainbow trout fed dietary E-L.r (30.66 U/mL) were significantly higher than in the control (22.33 U/mL) and E (17.00 U/mL) fed diets ($P < 0.05$). Regarding ACH50, the rainbow trout fed L.r (132 U mL^{-1}) and E-L.r (134 U mL^{-1}) diets had significantly higher values of the ACH50 as compared with those fed dietary E (119.00 U/mL) and control (110.67 U/mL). The plasma IgM changes revealed no significant differences between the treatments.

The gene expression of IL-1 and TNF-1α in the kidney was affected by both encapsulated and unencapsulated *Lact. rhamnosus* ATCC 7469 administrations as well as by spraying *Lact. rhamnosus* ATCC 7469 to the diet (Fig. 2). In this regard, rainbow trout fed E-L.r diet induced the highest expression of the IL-1 gene in the kidney tissue (0.25) compared to those fed the dietary control (0.06) and E (0.07) ($P < 0.05$). The expression of TNF-1α gene was significantly higher in fish fed L.r diet (0.73) as compared to those fed the control (0.35) and E (0.33) diets ($P < 0.05$).

Antioxidant Activities

Figure 3 reveals the results of antioxidant activities such as SOD and CAT of fish fed experimental diets. According to this figure, diet supplementation with probiotic, in any way used, significantly improved the SOD and CAT activities

Table 4 Body composition of rainbow trout fed different diets. Control basal diet (C), diet with empty microcapsules added (E), diet sprayed with *Lactobacillus rhamnosus* ATCC 7469 (L.r.), and diet with Lact. rhamnosus encapsulated added (E-L. r.)

Approximate analysis	Control	E	L.r	E-L.r
Protein (%)	15.75 ± 1.21 b	16.65 ± 0.95 ab	16.13 ± 0.88 ab	17.51 ± 0.56 a
Fat (%)	6.67 ± 0.76 a	5.88 ± 1.35 ab	5.71 ± 0.95 ab	4.82 ± 0.34 b
Ash (%)	1.89 ± 0.21	1.70 ± 0.55	1.90 ± 0.21	1.64 ± 0.24
Moisture (%)	74.91 ± 1.40	76.12 ± 0.56	76.42 ± 1.35	75.18 ± 0.14

Values are the means ± standard deviation of the six samples. Means without letter are not significantly different. Letters a, b, and c indicate significant differences in treatments, according to Duncan's multiple range tests ($P < 0.05$)

Table 5 Change to: blood biochemistry of rainbow trout fed different diets. Control basal diet (C), diet with empty microcapsules added (E), diet sprayed with *Lactobacillus rhamnosus* ATCC 7469 (L.r.), and diet with Lact. rhamnosus encapsulated added (E-L. r.)

	Control	E	L.r	E-L.r
Total protein (g/dL)	4.02 ± 0.64 b	4.28 ± 0.77 ab	4.20 ± 0.82 ab	4.98 ± 0.61 a
Albumin (g/dL)	2.45 ± 0.43	2.67 ± 0.55	1.99 ± 0.79	2.00 ± 0.40
Cholesterol (mg/dL)	185.67 ± 8.04 a	185.50 ± 9.60 a	156.50 ± 8.10 b	160.67 ± 10.33 b
Glucose (mg/dL)	59.66 ± 4.90	58.05 ± 8.57	57.33 ± 9.44	53.00 ± 8.42
Triglyceride (mg/dL)	78.33 ± 3.60	63.59 ± 4.82	59.00 ± 7.50	70.67 ± 6.51

Values are the means ± standard deviation of the nine samples. Means without letter are not significantly different. Letters a, b, and c indicate significant differences in treatments, according to Duncan's multiple range tests ($P < 0.05$)

($P < 0.05$). Thus, the fish fed dietary E-L.r had a significantly higher activity of SOD (203 U/mg protein) and CAT (528.33 U/mg protein) as compared to the rainbow trout fed the control diet ($P < 0.05$).

Challenge with *Y. ruckeri*

Microencapsulation of *Lact. rhamnosus* ATCC 7469 enhanced the resistance of rainbow trout against *Y. ruckeri* infection (Fig. 4). The highest post-challenge survival rate (70.36%) was observed in fish fed the E-Lr diet. The fish group fed the diet supplemented with free *Lact. rhamnosus* ATCC 7469 indicated 59.25% survival rate. Overall, the results indicated that the fish fed dietary L.r and E-L.r had a significantly higher survival ratio as compared with those fed the control and E diets.

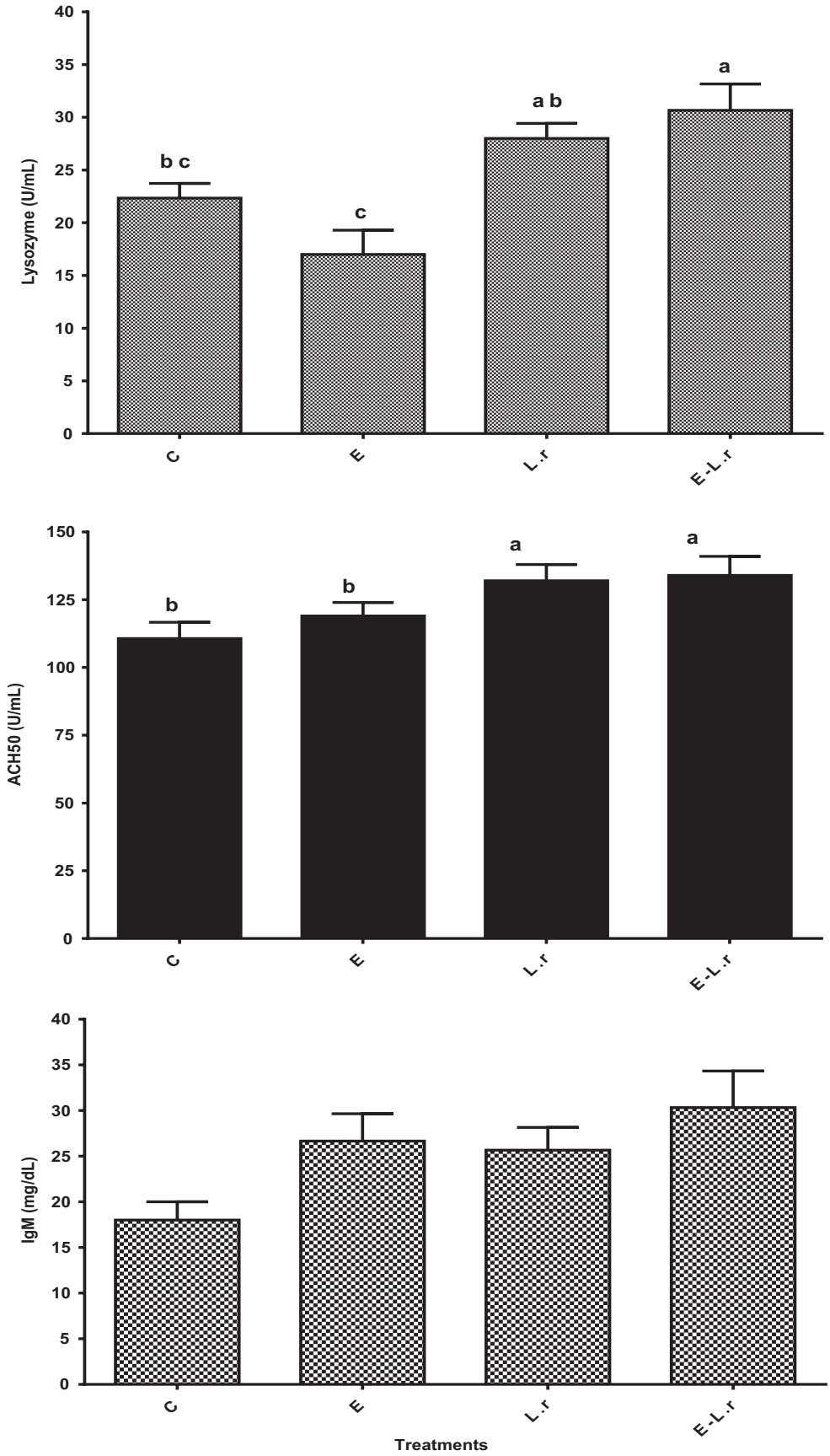
Discussion

Growth Performance

Probiotics are the most effective additives which provide a wide range of benefits such as growth promotion and immune system stimulation in the aquaculture industry. However, their efficiency can be negatively affected by poor bioavailability of viable microorganisms in the gastrointestinal tract. The microencapsulation of probiotic cells can increase the viability of probiotic bacteria during processing and delivery to the gastrointestinal tract [28, 29, 32]. Earlier studies have indicated the efficacy of administration of different species of *Lactobacillus* as a potential growth promoter in tilapia [21, 52], orange-spotted grouper (*Epinephelus coioides*), [53], roho (*Lobeo rohito*) [54], rainbow trout [35], and Caspian brown trout (*Salmo trutta Caspius*) [40]. Also, some studies have shown the role of different strains of *Lact. rhamnosus* in improving the growth in fish species [16–22]. However, there is no study on the administration of ATCC 7469 in aquaculture research. Although the administration of probiotics in aquatic animals has been broadly investigated, few works have focused on encapsulating probiotics [29]. In the present study, we showed that chitosan-alginate encapsulated *Lact. rhamnosus* ATCC 7469 improves the WG, SGR, and FCR of rainbow trout. Fish fed L.r diet had also significantly higher values of these parameters as compared with the control fish. We can conclude that probiotic *Lact. rhamnosus* ATCC 7469 could improve the growth performance in both sprayed and encapsulated forms. Interestingly, probiotic encapsulation had the highest growth performance effect. The best FCR value was observed in the fish fed encapsulated *Lact. rhamnosus* ATCC 7469 supplemented diet. Several in vivo works showed some significant positive effects of the encapsulation method to enhance the viability and/or the functional properties of the probiotic cells. Probiotics can improve nutrients' digestibility by increasing the activity of digestive enzymes, maintaining normal intestinal microbiota, and stimulating the synthesis of vitamins [6, 7, 38]. These improvements finally enhance growth performance and FCR [6, 7, 38]. Definitely, investigating digestibility, amino acids, and fatty acids profile of fish fed encapsulated probiotic supplemented diet can help us better understand the mechanisms. In our future work, we will investigate these factors to illustrate the possible reasons behind the enhanced growth performance and feed efficiency of rainbow trout fed the encapsulated probiotic supplemented diet.

Body Composition

Body compositions in the present study were affected by encapsulated probiotic where the fish fed dietary E-L.r had significantly higher and lower protein and lipid contents respectively. Although the chemical composition of aquatic species depends on internal factors and external factors (age, gender, size) and external factors (water quality, season, and geographical area), the main reason is usually the diet [55]. Our previous works also indicated changes in the body composition arising from the diet [56–59]. Parallel with the present study, some works indicated that inclusion of probiotics to the diet can increase the protein and reduce the lipid contents in the body [60–63]. We observed similar improvement of flesh quality (increased protein and decreased lipid) in the fish fed herbal medicine [64–66] and microalgae [67]. On the other


Lysozyme (U/mL)
40
35
30
25
20
15
10
5
0
b c
c
a b
a
C
E
L.r
E-L.r
ACH50 (U/mL)
150
125
100
75
50
25
0
b
b
a
a
C
E
L.r
E-L.r
IgM (mg/dL)
40
35
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Treatments

◀ **Fig. 1** Immune system parameters of rainbow trouts fed different diets for 60 days. Control basal diet (C), diet with empty microcapsules added (E), diet sprayed with *Lactobacillus rhamnosus* ATCC 7469 (L.r.), and diet with encapsulated *Lact. rhamnosus* added (E-L. r.). Values are means ± SE ($n = 9$). Values with different letters are significantly different ($P < 0.05$).

hand, some studies have reported elevated lipid in the body upon probiotic feeding [68, 69]. It was not clear how encapsulated *Lact. rhamnosus* ATCC 7469 could improve the flesh quality of rainbow trout in our study. Although we hypothesize that improving nutrient retention, digestibility, and absorption can explain this, more research is required.

Blood Biochemistry

Levels of plasma components in the blood are valuable tools for assessing the fish health [18]. In our study, biochemical blood parameters indicated that the diet supplemented with encapsulated *Lact. rhamnosus* ATCC 7469 increased the total protein in

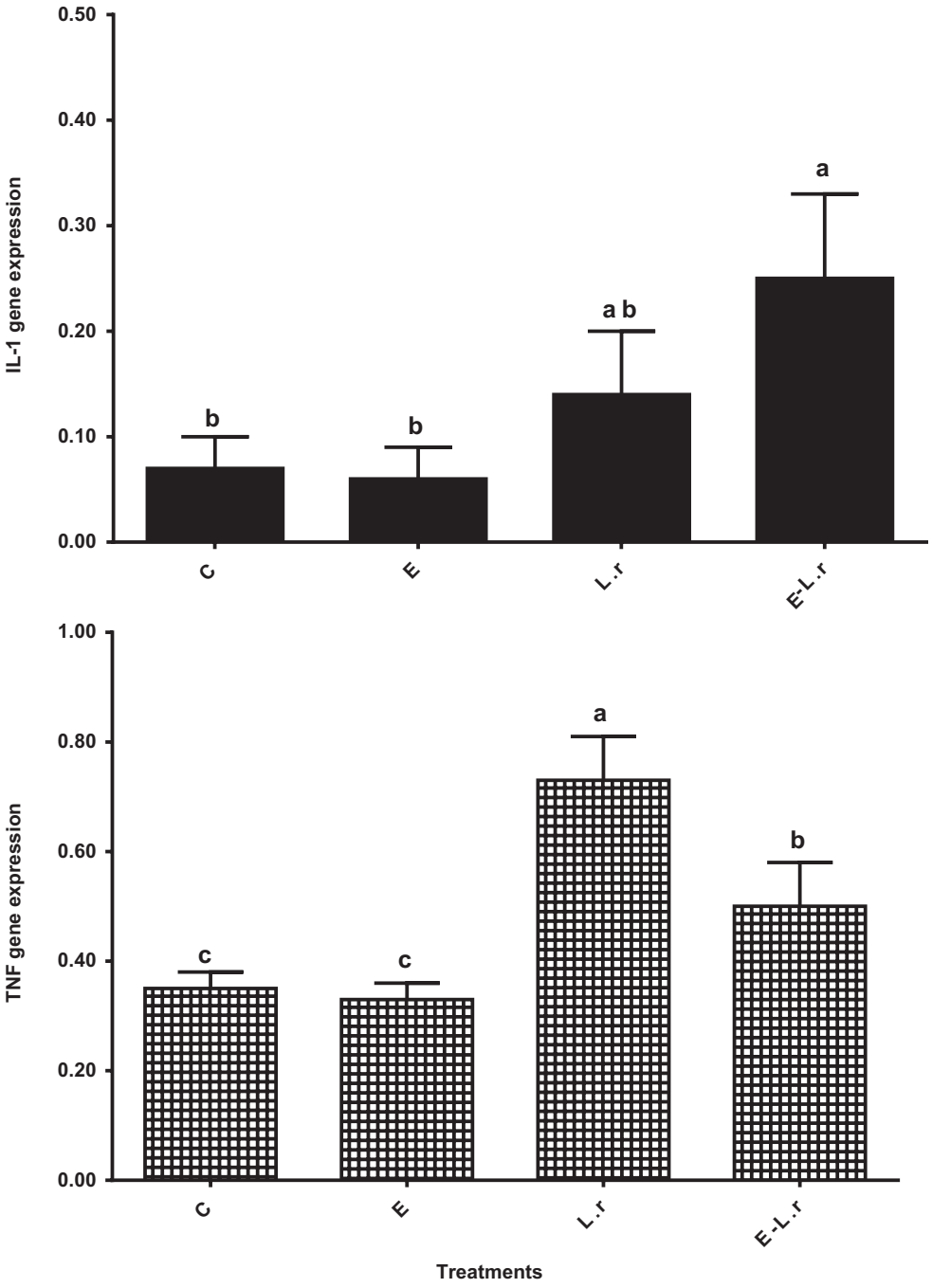

Fig. 2 Expression of immune system related genes in rainbow trouts fed different diets for 60 days. Control basal diet (C), diet with empty microcapsules added (E), diet sprayed with *Lactobacillus rhamnosus* ATCC 7469 (L.r.), and diet with encapsulated *Lact. rhamnosus* added (E-L. r.). Values are means ± SE ($n = 9$). Values with different letters are significantly different ($P < 0.05$)

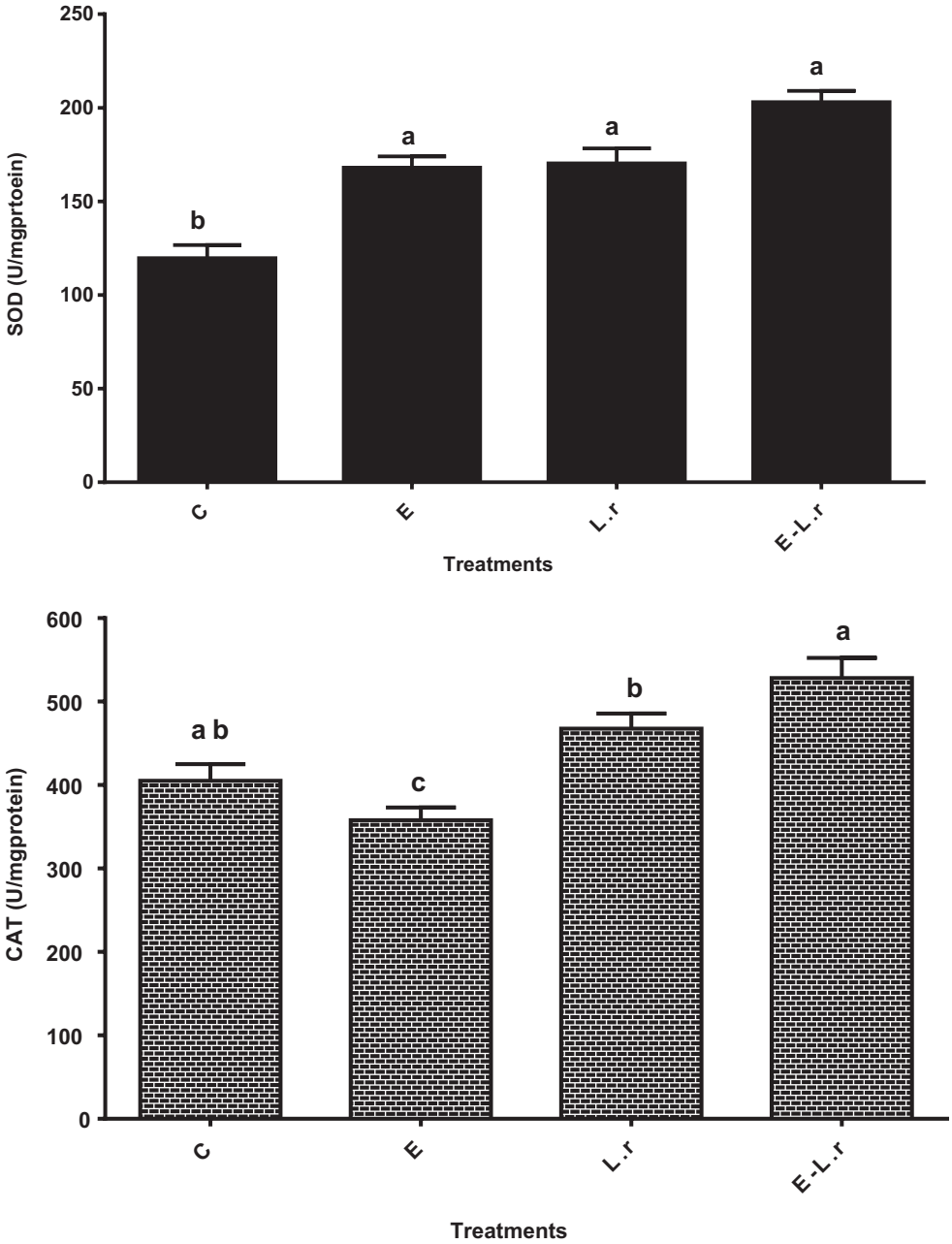

Fig. 3 Antioxidant system parameters of rainbow trouts fed different diets for 60 days. Control basal diet (C), diet with empty microcapsules added (E), diet sprayed with *Lactobacillus rhamnosus* ATCC 7469 (L.r.), and diet with encapsulated *Lact. rhamnosus* added (E-L. r.). Values are means ± SE ($n = 9$). Values with different letters are significantly different ($P < 0.05$).

the blood. Some researchers have reported that the rise in total proteins is an indicator of healthy condition as regards immunity in fish [7, 42, 70]. Other researchers reported a significant increase in the protein levels in the rainbow trout plasma fed diets including *Lact. rhamnosus* [18, 22] which were compatible with our study. Also, it was reported that the capsule including *Lact. rhamnosus* could activate the anabolic capacity of the hepatocytes to produce blood proteins [71]. Although plasma glucose levels remained unaffected, plasma cholesterol was significantly lower in the group fed free and encapsulated *Lact. rhamnosus* ATCC 7469 diets. Some studies previously showed that lipid components in the blood can be influenced by probiotic supplementation [8, 19, 70].

Immune Parameters and Immune-Related Genes

Immune response parameters such as lysozyme, ACH50, and IgM are considered as some appropriate indicators of the health status of fish under various nutritional and environmental conditions [72]. The results of the present study revealed that lysozyme and ACH50 were enhanced in the probiotic-treated fish groups, especially those receiving encapsulated *Lact. rhamnosus* ATCC 7469. Similarly, many studies indicated the positive effect of *Lact. rhamnosus* in improving the immune response [16–22]. We found no significant difference in the total IgM levels between different groups. Probiotics including

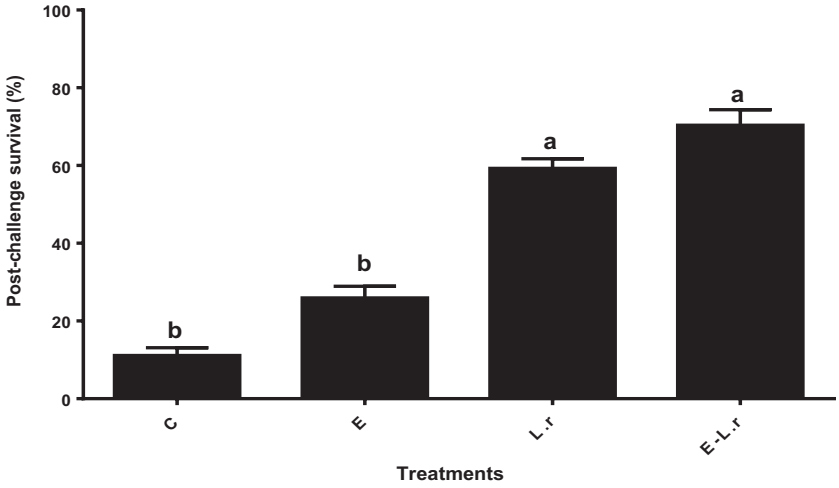

Fig. 4 Survival of rainbow trouts challenged with *Yersinia ruckeri*. Fish fed different diets for 60 days were intraperitoneally challenged with 107 CFU/mL of *Yersinia* and, after 14 days, the cumulative mortalities were recorded. Control basal diet (C), diet with empty microcapsules added (E), diet sprayed with *Lactobacillus rhamnosus* ATCC 7469 (L.r.), and diet with encapsulated *Lact. rhamnosus* added (E-L. r.). Values are means ± SE ($n = 18$). Values with different letters are significantly different ($P < 0.05$)

Lactobacillus spp. had increased lysozyme and ACH50 in fish [18, 19, 73, 74]. Lysozyme, one of the important bactericidal enzymes of innate immunity, is an important tool for fighting against infectious agents in the fish body [71]. One of the most important factors in innate immunity is ACH50 [16]. In the same way, some studies found that ACH50 was significantly higher in the fish fed different strains of *Lact. rhamnosus* diets [17, 53, 75]..

The expressions of two immune-related genes were evaluated in the fish fed encapsulated and sprayed *Lact. rhamnosus* ATCC 7469 supplemented diets. In the present work, IL-1 was significantly upregulated in the kidney of the fish fed dietary encapsulated *Lact. rhamnosus* ATCC 7469 and TNF-1α in the free probiotic group, when compared to the control. There is an agreement between our finding and previous studies, showing that the supplementation of probiotic bacteria in tilapia increased the expression of pro-inflammatory cytokines, including IL-1 and TNF-α [17]. Similarly, higher levels of IL10 and TNF-α induction have been reported in RAW264.7 murine macrophages treated with *Lactobacillus salivarus* microencapsulated into alginate/chitosan [76]. Furthermore, encapsulation of *Lactobacillus plantarum* into alginate/chitosan/alginate microcapsules induced secretion of TNF-1α and IL6 from macrophages and dendritic cells, proving the immunomodulatory effect of such a probiotic [77]. Higher plasma ACH50 corresponded with the TNF-α and IL-1 expression in the fish fed supplemented diet with the probiotic [17]. Overall, in the present study, encapsulated and sprayed *Lact. rhamnosus* ATCC 7469 diets could improve the immune response in both ACH50 and lysozyme, as well as gene expression level. Another point is that we can match the improvement of immune response and growth performance in rainbow trout fed E-L.r diets. Boosting immune response could indirectly enhance the growth performance of fish.

Antioxidant Activity

Superoxide anions, along with hydroxyl radicals and nitric oxides, are induced reactive oxygen species, supporting macrophages to kill microbial communities. Probiotics, as an immunostimulant, can enhance the phagocytic activity and the production of reactive oxygen metabolites by macrophages [78]. In fish species, SOD and CAT detoxify reactive oxygen species and they are thus considered as an indicator of antioxidant activity. SOD and CAT were significantly higher in the liver of fish fed E-L.r diet when compared with those fed the control. Similarly, in different species such as Japanese eel (*Anguilla japonica*) [78], red sea bream [79], common carp *Cyprinus carpio* [80], turbot (*Scophthalmus maximus*) [81], and tilapia [82] *lactobacillus* spp. could increase SOD and CAT activities. Based on the results, there was no significant difference in antioxidant activities of fish fed the encapsulated and sprayed probiotic to the diets. It means that antioxidant enzymes in rainbow trout are stable where an increase in the loading of probiotic could not enhance these enzymes. In other words, a specific level of probiotic was enough to enhance these enzymes beyond which this value did not change SOD and CAT activities. On the other hand, some authors found diminished SOD and CAT activities when the fish were fed a diet supplemented with probiotics [53]. However, most studies have reported enhanced antioxidant activities such as SOD and CAT in fish upon administration of probiotics to the diets rather than a decline.

Challenge with *Y. ruckeri*

Probiotics not only can promote the growth and boost immune response, they can also control bacterial infectious disease. The challenge test showed that *Lact. rhamnosus* ATCC 7469 increased the survival rates of rainbow trout against *Y. ruckeri*. The highest survival rate was obtained in free and encapsulated *Lact. rhamnosus* ATCC 7469 groups. In line with our findings, some studies found that probiotics increased disease

resistance in tilapia [14, 83] and rainbow trout [38]. In aquaculture studies, many works have reported potential of different strains of *Lact. rhamnosus* against *A. salmonicida*, *V. anguillarum*, and *F. psychrophilum* in rainbow trout [13], *E. tarda* [14], and *S. agalactiae* [15] in tilapia. Also, probiotics *lactobacillus* spp. in either free or bio-encapsulated form can improve the natural resistance and survival rate of larvae and postlarvae of fish [84]. Encapsulation causes the entrance of more bacteria into the intestines. Thus, encapsulated *Lact. rhamnosus* ATCC 7469 could enhance the immune response and eventually, caused a higher survival ratio in this group. However, the effect of encapsulation on resistance against bacteria and disease have rarely been investigated. Some studies indicated that the encapsulation of different probiotic bacteria and applying resistant starch as prebiotics and chitosan as a coating material significantly enhanced the survival rate of microorganisms in vitro [33, 85].

Conclusion

The current study showed how designing and developing a diet contains encapsulated probiotic in the aquaculture industry can improve growth and immune response. In general, encapsulated *Lact. rhamnosus* ATCC 7469 supplemented diets positively influenced the growth performance, body composition, blood biochemistry, antioxidant activity, and the immune system of rainbow trout. Furthermore, encapsulated *Lact. rhamnosus* ATCC 7469 more effectively enhanced the survival rate of rainbow trout after the challenge test with *Y. ruckeri.* As little works have been done to understand how encapsulated probiotics can affect the fish physiology, we would focus in the future research on the effect of encapsulation on digestibility, digestive enzymes, fatty acids, and amino acids profiles of fish.

Acknowledgments The authors gratefully acknowledge of the Tarbiat Modares University for the financial supported this research as a Ph.D thesis.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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